Noncomplementing diploidy resulting from spontaneous zygogenesis in *Escherichia coli*

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With the aim of understanding sexual reproduction and phenotypic expression, a novel type of mating recently discovered in *Escherichia coli* was investigated. Termed spontaneous zygogenesis (or Z-mating), it differs from F-mediated conjugation. Its products proved phenotypically unstable, losing part of the phenotype for which they were selected. Inactivation of a parental chromosome in the zygote is strongly suggested by fluctuation tests, respreading experiments, analysis of reisolates, and segregation of non-viable cells detected by epifluorescence staining. Some phenotypically haploid subclones were interpreted as stable noncomplementing diploids carrying an inactivated co-replicating chromosome. Pedigree analysis indicated that the genetic composition of such cells consisted of parental genomes or one parental plus a recombinant genome. Inactivation of a chromosome carrying a prophage resulted in the disappearance of both the ability to produce phage particles and the immunity to superinfection. Phage production signalled transient reactivation of such a chromosome and constituted a sensitive test for stable noncomplementing diploidy. Chromosome inactivation thus appears to be a spontaneous event in bacteria.

The persistence of genetic complementation in diploids isolated among artificial exfusants of *Bacillus subtilis* is the exception, not the rule. In most cases, one parental chromosome in identified diploids ceases to be transcribed, though continuing to replicate (Hotchkiss & Gabor, 1980; Fleisher & Vary, 1985; Guillén et al., 1983; Lévi-Meyrueis & Sanchez-Rivas, 1984; Le Derout et al., 1992; Grandjean et al., 1996a, b, 1998). These noncomplementing diploids (Ncds) can be mistaken for true haploids (Sanchez-Rivas & Lévi-Meyrueis, 1994 vs Hauser & Karamata, 1992). In such Ncds, genetic recombination between the active and the inactive chromosome is a rare event (Guillén et al., 1985).

Recent observations with *E. coli* led to the conclusion that total genetic mixing could occur and persist after contact between distinct populations of intact bacteria without any need to convert them into protoplasts beforehand. This phenomenon, called ‘illegitimate mating’ in the original report (Gratia, 1994), and later termed ‘spontaneous zygogenesis’ or Z-mating (Gratia & Thiry, 2003), was observed in an auxotrophic *E. coli* K-12 derivative (MG352). That strain, fully described in terms of characteristics and origin in the previous papers, was initially F− but acquired the ability to promote Z-mating upon infection with defective phage particles from *E. coli* clinical isolate 84SV. Examination of mixtures under the fluorescence or electron microscope using immunocytochemical methods revealed junctions between parental cells, at their poles, which appeared to be the possible sites of membrane fusion (Gratia & Thiry, 2003).

**INTRODUCTION**

The process of conjugation that is known in bacteria consists of the unidirectional and usually partial transfer of a copy of the replicating DNA molecule from a donor cell to a recipient cell. It differs fundamentally from the mechanism of sexuality in eukaryotes, where haploid germinal cells fuse to form diploid cells, initiating development of the offspring. However, the presumption of fusion between bacterial cells leading to the formation of diploids was proposed by Lederberg (1949) to account for various observations on prototrophs obtained in conjugation experiments with *Escherichia coli* K-12. Diploidy or persistent meroploidy after such crosses in *Escherichia coli* was confirmed by analyses of clonal pedigrees from isolated single cells (Zelle & Lederberg, 1951). Since then, complete diploidy has been achieved artificially by polyethylene-glycol-induced protoplast fusion in various bacteria (Fodor & Alfoldi, 1976; Schaeffer et al., 1976; Hopwood et al., 1977; Coetsee et al., 1979). This provides a very effective tool for studying genotype mixing, functional genetic complementation, genetic recombination and gene expression.

**Abbreviations:** Ap, ampicillin; ApR, β-lactamase encoding plasmid; GDA, Gratia–Deschuyteneer agar medium; LC/LCA, lactose-casein broth/agar; MA, minimal agar (variants with additives); NA, nutrient agar; Nx, nalidixic acid; Sm, streptomycin; Ncd, noncomplementing diploid; Szp−, spontaneous zygogenesis promoting; Z-mating, mating promoted by Szp+ bacteria.
In addition to this spontaneous zygogenisis-promoting (Szp⁺) property, MG352 displayed alternation between phenotype U3r and FhuA-specific agents (i.e. phages T1, T5, φ80vir and colicin M), and phenotype UFs, of sensitivity to all these agents (Gratia, 1994). In the same paper, successive crosses involving MG352 and genetically labelled F⁻ strains were described as having given rise to strains which, like MG352, existed in two forms, α or β. Such strains were interpreted as Ncds expressing either chromosome α (expressed in MG352 Ufr) or chromosome β (from the F⁻ partner with a UFs phenotype) respectively. MG388α/β is one of such strains marked at 12 loci from carB at 1 min to met at 90 min.

Therefore, unlike what is usually thought, the genetics of bacteria has not yet revealed all its secrets, some of which seem to be reminiscent of eukaryotic events such as sexual reproduction or even ‘epigenetics’. The object of this paper is to confirm the existence of noncomplementing diploidy in the descendants of Z-mating products. It appears that inactivation of a chromosome or a plasmid may occur frequently. Z-mating products could be selected for as long as they still expressed both parental chromosomes carrying distinct auxotrophic mutations. They could form colonies on minimal agar. In the course of subsequent growth, markers of one entire chromosome became silenced. When still diploid, cells could not be selected as such and behaved as phenotypically haploid. True haploid segregants carrying the inactive chromosome died and were not detectable unless samples were examined by epifluorescence staining. The discovery of this phenomenon not only provides a new approach to the study of sexual reproduction in bacteria, but opens up new avenues of research, such as the regulation of the co-replication of two chromosomes and the inactivation of whole DNA molecules.

**Table 1. F⁻ and Szp⁺ strains of E. coli K-12**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Conjugational phenotype</th>
<th>Source or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1485/Nx</td>
<td>trpA,B his nalA</td>
<td>F⁻</td>
<td>N. Franklin; NalA⁻ mutant</td>
</tr>
<tr>
<td>A69</td>
<td>ara Δ(pro–lacZ) rpsL</td>
<td>F⁻</td>
<td>Derived from P90C (Gupples et al., 1990); from R. D’Ari through D. S. Thaler†</td>
</tr>
<tr>
<td>C202H19 (or H19)</td>
<td>lacY trpE recA rpsL mal</td>
<td>F⁻</td>
<td>Grätia (1973); derived from P678</td>
</tr>
<tr>
<td>MG351</td>
<td>carB leu galK [z] recA rpsL metB</td>
<td>F⁻</td>
<td>D. Charlier†</td>
</tr>
<tr>
<td>MG388 x/β</td>
<td>fhuA pro lacY argG rpsL (z)/carB leu galK [z] recA rpsL metB (β)</td>
<td>Szp⁺</td>
<td>Grätia (1994)</td>
</tr>
<tr>
<td>MG391</td>
<td>fhuA pro lacY rpsL</td>
<td>Szp⁺</td>
<td>This study: ArgG⁺ revertant of MG388x</td>
</tr>
<tr>
<td>MG393 x/β</td>
<td>fhuA pro lacY rpsL [808hr]/trpA,B nalA</td>
<td>Szp⁺</td>
<td>This study</td>
</tr>
<tr>
<td>MG401</td>
<td>lacY rpsL metB</td>
<td>Szp⁺</td>
<td>Grätia &amp; Thiry (2003)</td>
</tr>
<tr>
<td>MG403</td>
<td>trpA,B nalA</td>
<td>Szp⁺</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Mutation of thiamine requirement is not indicated, as this vitamin was present in all media.
†D. S. Thaler, Sackler Laboratory of Molecular Genetics and Informatics, Rockefeller University; L. D. Charlier, Laboratory of Microbiology, Vrije Universiteit te Brussel.

**METHODS**

**Bacterial strains.** Table 1 lists the E. coli K-12 strains used as Z-mating-promoting (Szp⁺) clones and the F⁻ strains used as partners in crosses. All strains carry various auxotrophic mutations that did not revert under the conditions used (less than 10⁻⁷ per plated c.f.u.). Some strains ferment lactose; others do not (strains MG388x, MG391 and MG401 have inherited an irreversible lacY mutation from MG301; A69 and derivatives carry a lacZ–pro deletion). Almost all strains are resistant to streptomycin (Sm) by rpsL mutation or resistant to nalidixic acid (Nx) by nalA mutation. The most commonly used Szp⁺ strain, MG392, was derived by Z-mating between MG391, an Arg¹ revertant of the Szp⁺ strain MG388x, and F⁻ 1485/Nx. The Szp⁺ strain MG401 was created by exposing F⁻ strain MG301 to lysates of a lysogenic E. coli clinical isolate (84SV) induced to phage development (Grätia & Thiry, 2003). MG403 was a Szp⁺ recombinant derivative resulting from a cross between MG401 and F⁻ 1485/Nx. ApR derivatives of MG391 and MG392, resistant to 10 µg ampicillin ml⁻¹, were constructed by transformation with a plasmid DNA using the heat-shock CaCl₂ method (Sambrook et al., 1989). The Ap⁺ plasmid was a derivative of the non-selftransferable pMMB66 (Fürste et al., 1986) carrying the bla⁺ gene encoding clavulanate-sensitive β-lactamase (kindly provided by Dr M. Knight, Plant Physiology Department, University of Oxford, UK).

**Media and chemicals.** The media used here have been described previously (Gratia, 1994; Gratia & Thiry, 2003): M9 buffer, Lennox broth (LB), nutrient agar (NA), LB agar, minimal agar (MA; Simsoms-citrate agar with added lactose, instead of glucose, plus casein acid hydrolysate (‘peptone 5’; Gibco), when parents differed in lactose fermentation and were deficient for nutrients not included in peptone 5), and Gratia–Deschuyteneer agar medium (GDA; see below). If necessary, specific substances were added to these media. As several strains were auxotrophic for thiamine, this vitamin was systematically included in all synthetic media at the final concentration of 1 µg ml⁻¹. MacConkey agar, supplemented with lactose, galactose, maltose or arabinose at a final concentration of 1% (w/v) when pouring, was used in fermentation tests. The chemically defined GDA medium (Gratia & Deschuyteneer, 1998; Gratia & Thiry, 2003), which contained lactose used by one of the mating partners,
a limiting amount of glucose and nutrients, plus a pH indicator (bromothymol blue), allowed the visualization of phenotypic switching. When a LacZ strain was involved in matings, the reagent X-Gal was used on M9 agar with added 10 g Bacto-peptone (Difco) l⁻¹ in the presence of IPTG (an inducer of β-galactosidase in the LacZ+ partner), according to Miller (1972). For some tests, media were supplemented with 200 μg streptomycin sulphate ml⁻¹ or with 20 μg nalidixic acid ml⁻¹ for an overnight incubation. Minimal liquid medium lactose-casein broth LC was used in some experiments: M9 buffer plus 5 g lactose l⁻¹, 50 mg ‘peptone 5’ 1⁻¹ and 1 μg thiamine ml⁻¹. Solutions of 50 μg nalidixic acid ml⁻¹ were also used for spot testing when noncomplementing diploidy was sought in strains in which an inducible prophage could be transiently expressed. Agar medium (NA, MA or LCA) was supplemented with 5 μg ampicillin ml⁻¹ for the selection of bacteria expressing the bla⁺ gene of plasmid ApR.

Z-mating test and experiments. The ability of newly isolated clones to promote Z-mating was detected as follows. Drops containing 10⁶–10⁸ cells of a strain to be tested for the Szp⁺ character and of an F- tester were deposited on a medium where unmixed strains were unable to grow, so as to overlap. The bacteria were starved for 1 h in M9 to limit residual growth and cross-feeding. The test was considered positive when colonies appeared within a 3 day incubation at 37 °C in the zone where tested and tester bacteria were mixed. Control of Z-mating was provided by comparison with isogenic F⁻ strains unable to promote any form of mating.

Exponentially growing cultures of confirmed auxotrophic Szp⁺ clones were mixed with bacteria of an F⁻ strain bearing different auxotrophic markers according to Gratia & Thiry (2003). Samples of 20 min mixtures incubated at 37 °C, concentrated by centrifugation when needed, were plated on MA or LCA. Colonies were purified on the same medium as used for selection.

Analysis of Z-mating products. This analysis focused on colonies formed on any medium enabling visualization of functional genetic complementation. Two purification steps were carried out as described by Gratia & Thiry (2003). When instability persisted and changes occurred after reisolation, bacteria were restreaked for further analysis. Several different colony types were observed on GDA: rough white (rw), smooth yellow (sy) and sectored rw/sy (Fig. 1a). The frequency of each colony type was estimated. Some colonies were picked up for incubation in broth and analysis with respect to (a) response to nutrients on MA, (b) sensitivity to antibiotics and to UV (UV sensitivity signalled a RecA⁺ phenotype) on NA, (c) phage sensitivity on LB agar plates, and (d) fermentation of various sugars by diluted samples dropped on MacConkey agar, or by isolated colonies after restreaking (Fig. 1b). Because of its high sensitivity and the intense blue stain diffused by the few LacZ⁺ cells upon induction by IPTG, X-Gal was especially useful for detecting the presence of an active lacZ⁺ allele, even a rare one, or when expression was transient (Fig. 1c). The nature of true genetic recombinants was unambiguously shown when isolates that expressed a recombinant phenotype in terms of nutritional abilities were fully sensitive to streptomycin or a virulent phage, to which one parent was resistant.

Single cell isolation. The method initially devised by Lederberg (1954) was used to isolate single cells derived from presumptive diploid heterozygotes. The content of colonies issuing from a purified clone previously shown to sequester a few cells of another type was resuspended in a small volume of distilled water. The suspension was strongly vortexed and diluted in LB so as to lower the cell density to 1–5 × 10⁶ viable cells ml⁻¹. Microdrops of about 0.1 μl were deposited within a layer of paraffin oil on slides and were examined under phase-contrast (Reichert microscope, × 400 magnification) to detect individual cells. After a brief incubation of the slides at 37 °C, a bacterium isolated from one microdroplet initially found to contain only one single cell was analysed.

Fig. 1. Examples of sectored colonies in heterogeneous populations of isolates from Z-mating products. Sectored colonies on: (a) GDA with Lac⁺ smooth, yellow and denser sectors or buds within rougher white colonies of Lac⁻ bacteria (rw/sy); (b) MacConkey-lactose agar with Lac⁺ red colonies or sectors; (c) X-Gal plates, where bacteria expressing the lacZ⁺ allele are stained.

Enumeration of non-viable cells by fluorescence microscopy. Estimation of non-viable cells in cultures was based on an epifluorescence staining method using a mixture of the nucleic-acid-binding stains SYTO 9 dye and propidium iodide (viability kit L7012 developed by Molecular Probes, 1995). SYTO 9 stained cells green, while propidium iodide penetrated cells whose membrane has been damaged, staining them red. As recommended by Boulos et al. (1999), the samples were incubated with the staining mixture for 15 min at room temperature in the dark. Slides were examined under a Zeiss fluorescence microscope (magnification × 400) equipped with a halogen lamp, a 546 nm excitation filter and a 590 nm barrier filter. Red and green cells were counted on 2–4 microscopic fields of each of three preparations of the same bacterial suspension, i.e. on a total of 6–12 fields, each showing 30–100 well-separated cells. The recorded
values gave the mean frequency of non-viable cells among the total amount of counted cells, with a standard deviation from 0.03 to 0.12.

**Bacteriophages and lysogenic testing.** Bacteriophages λvir, φ80vir or TS, hybrid φ80h/λvir or φe (Gratia, 1989) were used to check for the presence or absence of corresponding receptor proteins, the products of lamb, fhuA, and fig respectively. Temperate Fig-specific φ80h/c+ was used to lysogenize the FhuA− Fig− form of the heterozygotic strain MG388, while φ80 could infect and lysogenize the β form of that strain, which was FhuA+ Fig+. (λ was already present in the b chromosome expressed in this form). In both cases, lysogenization occurred through insertion of the phage genome in att80 (Gratia, 1989). Clear c mutants of λ and φ80 (or φ80h/λ) were used to check for immunity to superinfection with λ and φ80 respectively, and thus the presence of the corresponding prophage in the bacteria studied. The text supplies details concerning experiments aimed at detecting various events where prophage development was involved, especially in the rare case of conversion of one form to another in a strain suspected of being a ly−. Usually these experiments involved a streaking experiment on plates with a selective medium allowing limited growth for 1 h at 37°C, followed by a microscopic examination of samples in a parallel culture. Then cells were distributed into tubes at the average rate of a maximum of one single cell per two tubes. Aliquots from tubes displaying growth were plated on nutrient agar with added streptomycin (NA + Sm) or nalidixic acid (NA + N) to count the parental types. The number of cultures actually displaying a mixed population (9 tubes) exceeded the maximum number of tubes expected, from Poisson distribution, to have received cells of both parental types P1 and P2 in the absence of mating, i.e. at most 6 tubes. To these 9 observed tubes with P1 and P2 cells, one must add 6 of the 18 tubes containing an apparently pure P1-type population after 40 h, which were found to contain also P2-type cells after a 120 h incubation (Table 2, last line). A total of 15 instead of 6 tubes with the two parental types indicates a probability for this result to be significantly different.

### RESULTS

**Evolution of Z-mating products**

Some of the products of crosses between auxotrophic F− strains of *E. coli* K-12 and Szp + strains such as MG388 or MG401 had acquired the ability, upon mixing with F− partners, to form colonies on a medium that does not support the growth of either parent. Szp− strains MG392 and MG403 were generated in this way.

When the mating mixture was directly plated on GDA, the frequency of sectored colonies, which were possible mating products detected on a medium providing a selective advantage, was much higher (up to 20%) than the frequency of selected clones (about 10−4 per minority parent). This was not observed when other F− collection strains were mixed. The biparental phenotype in these sectored colonies might merely reflect a peculiar ability to stick to each other without being meaningful in terms of zygogenesis. The following experiments were performed to address whether sectored colonies are derived from a transient complementing diploidy which did not persist long enough to allow colony formation.

A first series of experiments was carried out with the aim of estimating the frequency of zygote formation, assessing the failure of persistent complementing diploidy and revealing phenotypic switching. Among them, the following fluctuation test was performed after a cross between strains MG392 (P1 in Table 2; Try− Na+) and MG391 (P2; Pro− Lac− RpsL−). The mixture was co-incubated for 20 min as usual, and a strongly vortexed sample was diluted in a medium allowing limited growth for 1 h at 37°C. This treatment was required for the passage from possible cell aggregation to cells moving freely, among which possible zygotes would have already been formed, as detected by microscopic examination of samples in a parallel culture. Then cells were distributed into tubes at the average rate of a maximum of one single cell per two tubes. Aliquots from tubes displaying growth were plated on nutrient agar with added streptomycin (NA + Sm) or nalidixic acid (NA + N) to count the parental types. The number of cultures actually displaying a mixed population (9 tubes) exceeded the maximum number of tubes expected, from Poisson distribution, to have received cells of both parental types P1 and P2 in the absence of mating, i.e. at most 6 tubes. To these 9 observed tubes with P1 and P2 cells, one must add 6 of the 18 tubes containing an apparently pure P1-type population after 40 h, which were found to contain also P2-type cells after a 120 h incubation (Table 2, last line). A total of 15 instead of 6 tubes with the two parental types indicates a probability for this result to be significantly different.

### Table 2. Fluctuation test: fate of individual c.f.u. in Z-mating mixtures

<table>
<thead>
<tr>
<th>c.f.u. titration (per ml)*</th>
<th>Separate P1 suspensions</th>
<th>P2</th>
<th>Mixtures on NA</th>
<th>NA + Sm</th>
<th>NA + Nx</th>
<th>Inoculum (c.f.u. in 250 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.9 ± 0.1 × 10⁶</td>
<td>6.0 ± 0.4 × 10⁶</td>
<td>6.8 ± 0.6 × 10⁶</td>
<td>5.1 ± 0.1 × 10⁶</td>
<td>1.6 ± 0.1 × 10⁶</td>
<td>1.4 ± 0.1 × 10²</td>
</tr>
</tbody>
</table>

*C. This treatment was required for the passage from possible cell aggregation to cells moving freely, among which possible zygotes would have already been formed, as detected by microscopic examination of samples in a parallel culture. Then cells were distributed into tubes at the average rate of a maximum of one single cell per two tubes. Aliquots from tubes displaying growth were plated on nutrient agar with added streptomycin (NA + Sm) or nalidixic acid (NA + N) to count the parental types. The number of cultures actually displaying a mixed population (9 tubes) exceeded the maximum number of tubes expected, from Poisson distribution, to have received cells of both parental types P1 and P2 in the absence of mating, i.e. at most 6 tubes. To these 9 observed tubes with P1 and P2 cells, one must add 6 of the 18 tubes containing an apparently pure P1-type population after 40 h, which were found to contain also P2-type cells after a 120 h incubation (Table 2, last line). A total of 15 instead of 6 tubes with the two parental types indicates a probability for this result to be significantly different.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Total</th>
<th>With growth</th>
<th>Expected no. of tubes to have received a number of c.f.u. equal to</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>With expected mixture P1 + P2</th>
<th>6</th>
<th>Displaying mixed population</th>
<th>9</th>
<th>With clones P1 later throwing off P2 cells†</th>
<th>6</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>248</td>
<td>104</td>
<td>79</td>
<td>21</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean values (± SD) of six measurements, i.e. two titrations in triplicate.
†Plating of the remaining volume of 120 h cultures on selective medium MA + proline + Sm.
(0.05 > P > 0.02) from the expected number of tubes in the case of random distribution of parental cells unable to form diploid cells. Therefore, 6.5% of the 139 distributed c.f.u. would have been biparental zygotes. This is probably an underestimate, since clones displaying one parental phenotype can eventually throw off cells of the other parental type, even after serial subculturing on a medium counterselective for the latter. This assertion is supported by the number of prototrophs expected to have been produced. At 1 h intervals, most of the plates were subjected to respreading. The behaviour of a true prototrophic recombinant was examined in parallel. Fig. 2 shows how the number of prototrophs evolved in one of these experiments. In the case of the true recombinant, the logarithm of the colony count increased linearly with time (upper curve). On the other hand, plates seeded with samples of Z-mating mixture displayed an abnormally low and very variable number of colonies (lower part of the graph).

Table 3 shows the results of a similar respreading experiment involving the same strains, except that P1 (MG392) harboured the ApR plasmid. The conditions of plating of the mating mixture samples on MA + Ap were such that the probability of more than one ampicillin-resistant, prototrophic colony appearing was low. By the end of the experiment, a number of colonies, corresponding to 6–7 generations, had appeared on the plates seeded with the true recombinant, which was used as a control. By contrast, plates seeded with the mating mixture displayed a low and very variable number of colonies.

Both respreading experiments show that the expression of both parental genomes does not necessarily persist in the progeny of a complementing diploid for long. This highlights the instability of complementing diploidy and suggests that the number of diploids initially formed could be much higher than indicated by the number of colonies visible on the selective medium. The question arises whether this instability is due to segregation of truly haploid cells or unequal expression of positive alleles in stable heteroploids.

**Table 3. Respreading experiment: low persistence of functional complementation in products of Z-mating between MG392 (ApR) (P1) and MG391 (P2)**

<table>
<thead>
<tr>
<th>Seeded sample</th>
<th>No. total c.f.u. plated on MA + Ap*</th>
<th>No. Trp+ Pro+ ApR colonies on plates†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not respread</td>
</tr>
<tr>
<td>P1 : P2‡</td>
<td>1</td>
<td>1 (2); 2 (1)</td>
</tr>
<tr>
<td>P1 × P2§</td>
<td>3 × 10³</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

*Both preparations were diluted in M9 + 0.5% lactose and, after a 40 min incubation, 100 μl aliquots were spread over MA + Ap plates. The cell density of the plated suspension was such that only one prototrophic Z-mating product (or at most two) could be formed on a plate (accordingly, more than 6/10 plates did not show any colony at all). Some plates were respread after a 6 h incubation at 37°C. †Colonies were counted after 48 h. The number of plates where colonies appeared is shown in parentheses. ‡True and stable prototrophic ampicillin-resistant recombinant; generation time about 50 min.
§A 20 min mating mixture of P1 and P2. Cell density: 1·2 × 10⁶ c.f.u. ml⁻¹; ratio of P1 to P2 = 2/3.
Higher frequency of non-viable cells as an argument in favour of chromosome inactivation

From the observations reported up to now, one can expect that soon after Z-mating there might be inactivation of one parental chromosome. If Z-mating products threw off haploid cells carrying an inactive chromosome, these would be non-viable. Assessment of the loss of viability of bacteria must be done on an individual basis. Fluorophores sensitive to membrane potential can distinguish between viable bacteria able to form colonies and non-viable cells undetectable by other means. This is particularly required in studies of heterogeneous populations such as during the stationary phase, so was expected to be useful in the present investigation.

Z-mating between Szp+ strain MG392 and F− partner A69 was examined with respect to the amount of non-viable cells in various stained preparations: (i) parents of the cross, (ii) 30 min mating mixtures and (iii) Z-mating products formed on LCA, before and after incubation in lactose-casein broth LC. If there were nothing noticeable, one should expect the same frequency of non-viable cells, coloured red when examined using a fluorescence microscope, in mating mixtures as in the suspensions of the parents. This is not the case: it was higher. This frequency was low for the F− parent A69 (0±055±005) and hardly higher for the Szp+ partner MG392 (0-11±0-015), whether or not they were incubated for 21–24 h. On the contrary, the mating mixture showed a significant increase of that frequency, i.e. 0-25±0-02 before incubation, and 0-41±0-04 after incubation under the same conditions as the parents. Afterwards, when the mating mixtures were reincubated in the same medium for an additional period, there was a progressive decrease down to the values of the unmixed parental cultures (0-20±0-06 vs 0-06 for A69 and 0-04 for MG392).

Small colonies formed by Z-mating within 2 days on LCA were picked up and put in the corresponding lactose-casein broth LC. Eleven non-purified isolates, which differed by the relative frequency of parental and recombinant types in each of them as shown before (Gratia & Thiry, 2003), were found to split into three classes according to the frequency of cells coloured red: (i) 0-12±0-007, (ii) 0-22±0-015 and (iii) 0-31±0-01. Upon further incubation in LCA, which gave a selective advantage to a single recombinant type, viable cells went on to multiply, so the frequency of dying cells declined to about 10% or even less after sub-culturing (0-073±0-025; total of 10 fields).

If the differences in the frequency of dying cells reflected well the segregation of haploid cells carrying an inactive chromosome, the results indicate that this segregation began during the first stages of the evolution of Z-mating products. Observations and experimental results described in the following sections provide complementary evidence of chromosome inactivation in the descendants of clones.

Quantitative discrepancies in the analysis of phenotype switching

As expected, quantitative differences emerged from the scoring system. The question is whether the observations will help to foresee what happens all along the succession of events. Therefore particular attention was paid to experiments such as the following.

A mixed suspension of MG391 and MG392 was directly plated on various indicator media. Samples from each of 16 subcultures displaying a mixed type on GDA were again plated on GDA, which was permissive for either Lac+ MG392-like or Lac− MG391-like bacteria and allowed the composition of the colonies at the end of their growth to be determined. At the same time, samples were plated on NA+Sm for the streptomycin resistance of MG391 and NA+Nx for the nalidixic acid resistance of MG392. It was anticipated that, after incubation, the number of colonies found on GDA would be equal to the number of colonies on NA+Sm and NA+Nx combined. This was not observed: the number of colonies on GDA was higher. This suggests that at the time of plating a highly variable fraction of the mixed population [mean of 9±65±11-3% (16)] was sensitive to both antibiotics and might consist of complementing diploids expressing dominant wild-type alleles rpsL+ and nalA+.

The analysis went one step further as follows. Colonies were counted in 12 samples plated on GDA, NA+Sm and NA+Nx. As checked by replica-plateing, all the smooth, yellow (sy) colonies formed on GDA corresponded to Lac+ NaLA− MG392-like bacteria and the rough, white (rw) colonies were those of Lac− RpsL− MG391-like bacteria. However, there was a discrepancy in the ratio between the parental-type colonies when calculated in different ways. The ratio obtained by dividing the number of sy colonies by the number of rw colonies was greater than the ratio given by dividing the number of colonies on NA+Nx by the number of colonies on NA+Sm, by a coefficient of 1-29±0-33 (12). This difference is significant, as the Student t test for 12–1 counts is 0-01 (1-29–1)/(0-33)/12)=3-04, i.e. higher than 2-2. This could mean that on lactose-containing GDA the ‘doubly sensitive’ entities present in the mixed population preferentially evolved into Lac+ NaLA− forms rather than Lac− RpsL− forms. Alternatively, growth on GDA might somehow favour switching of the Lac− RpsL− to the Lac+ NaLA− phenotype, thus producing sectored colonies instead of parental type rw colonies.

Evidence of persistent silent chromosome in purified isolates from Z-mating-derived products

The case of the Ufr/s strain MG352 and of the α/β strain MG388 as Ncds expressing only chromosome a in the Ufr or α form and chromosome b in the Ufs or β form was described in detail in previous work (Gratia, 1994). It was
imperative to extend such observations to the Z-mating products in general.

The above-described cross between Pro− Lac− RpsL− MG391 and Trp− Nal− MG392 is reconsidered as an example. From 80 Pro− Trp− clones examined, 239 subclones were analysed in detail. Among them, 16 were found to be biparental after reisolation, and 69 were also mixed and consisted of one parental and one recombinant type. The parental type was that of MG391, but after restreaking on permisive NA medium, some reisolates of that type still persisted in throwing off Lac+ Trp+ Nal− RpsL− recombinants, one of them being stable as its culture contained 1 × 10−5 recombinant per plated c.f.u.

Another example of a stable Ncd was the strain MG403, obtained after a cross between the Szp+ strain MG401 (LacY− RpsL− Met−) and the F− strain 1485/Nx (Trp− His− Nal−). It exhibited most of the phenotype of its F− parent and had become Szp+. It was crossed with the F− strain A69 (Ara− Pro− LacZ− RpsL−). A subclone exhibiting the parental A69-like phenotype was scored as cured and haploid. However, some of the white LacZ− colonies it formed on X-Gal medium gave rise, after a long incubation, to blue sectors or buds (See Fig. 1c). These were due to the activity of the lacZ+ allele inherited from MG403. Such buds or papillae were never observed in A69 colonies plated under the same conditions. Reversion to the LacZ+ phenotype was excluded since the mutation in A69 is a lacZ− proB deletion.

The A69-like isolate was subjected to single-cell isolation in order to confirm its noncomplementing diploidy. Only one of the microdroplets examined by phase-contrast microscopy was found to contain a cell; the 16 other microdroplets from the same suspension were empty and remained empty after incubation. A fluctuation test was performed with the single-cell isolate. It clearly showed segregation of recombinant Lac+ Pro− cells (still Ara− RpsL−) mimicking a spontaneous mutation (Table 4). The late and infrequent switch in such isolates rules out the possibility of sticky parental cells.

### Plasmid behaviour in Z-mating products and in their descendants

Resistance to ampicillin encoded by the plasmid-carried bla+ gene provided the opportunity to analyse the evolution of a non-essential genome under different conditions of selection. Particular attention was devoted to crosses involving the ApR plasmid carried by either or both MG391 and MG392 parents.

When mixtures of about 10⁶ cells of MG391 and an ApR derivative of MG392 were plated on NA plus 5 μg ampicillin ml⁻¹, the frequency of resistant colonies was lower than that expected, based on the c.f.u. counts of MG392 (ApR), i.e. 0.7 ± 0.1 per c.f.u. of that strain plated alone on the same medium without ampicillin. The same was observed when this mixture was plated on MA plus tryptophan and 10 μg ampicillin ml⁻¹ for the selection of the resistant partner MG392 (ApR), i.e. the relative frequency of resistant colonies on this medium was 0.63 ± 0.1.

The formation of complementing Z-mating products was also affected by the presence of added 1 μg ampicillin ml⁻¹ even when both parents were ApR derivatives: the frequency of prototrophic colonies counted on MA was about two times lower on MA + Ap. However, all prototrophic clones contained resistant cells whatever the selection procedure. Subclonal analysis revealed that such resistant clones could throw off sensitive bacteria. On the other hand, sensitive clones did transiently display a low degree of resistance and, upon reisolation, yielded truly resistant subclones. In all tested isolates, the resistance spectrum was as expected because of the specificity of the plasmid-encoded enzyme, i.e. resistance was to ampicillin or penicillins, not to piperacillin or cephalosporins.

The loss of resistance could therefore be interpreted as signalling the absence of functions of the plasmid rather

### Table 4. Fluctuation test in a pure cell line of a parental Lac− A69-like reisolate from a Z-mating product between MG403 and A69: re-expression of silent lacZ+ proB+ recombinant genome

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Vol. of cultures</th>
<th>Vol. of samples</th>
<th>Inoculum per culture</th>
<th>Final no. of bacteria per culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0-5 ml</td>
<td>0-02 ml</td>
<td>3</td>
<td>2·5 × 10⁷</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of Lac+ Pro+ colonies†</th>
<th>Mean/Ni per sample</th>
<th>Variance</th>
<th>Switch rate (ln2 × &lt;1·1/1 × 10²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1·1 × 10⁻⁵§</td>
<td>6·3 × 10⁴</td>
<td>&lt;7·6 × 10⁻⁶‖</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11–30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31–100</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>101–200</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†72 h incubation in M9 + 10% LB.

‡Selection on minimal lactose agar (Simmons-citrate agar with added 5 g lactose l⁻¹ and 1 μg thiamine ml⁻¹).

§Formation of Lac+ buds or sectors in part of the Lac− colonies upon incubation on X-Gal plates.

‖Mean number of Lac+ Pro+ per amount of c.f.u. in a sample [Nt (the number of bacteria in the tested sample of the final culture) = 1 × 10⁶].

If one assumes a Poisson distribution of Lac+ colonies among the independent cultures, their mean number should be lower since samples from more than half the cultures did not contain cells having switched to Lac+ Pro+ phenotype.
than its cure. The transient eclipse of plasmid expression can be compared to the silencing of a chromosome in an Ncd.

**Evidence for an ancestral silent genome in an Ncd**

A remarkable phenomenon observed among reisolates of a Z-mating product attracted particular attention, as it was reminiscent of atavism in eukaryotes. MG403, which inherited the phenotype Lac\(^+\) Trp\(^-\) Nal\(^-\) of its F\(^-\) parent 1485/Nx and the Szp\(^+\) character of MG401 as mentioned above (see third paragraph of the section ‘Evidence of persistent silent chromosome...’), was crossed with the F\(^-\) Lac\(^-\) TrpE\(^-\) RecA\(^-\) strain H19. A Z-mating product that kept the same phenotype as its F\(^-\) parent H19 and acquired the character Szp\(^+\) of MG403 was in turn crossed with the F\(^-\) RecA\(^-\) strain MG351, from which MG388 is derived. A RecA\(^-\) \(6\) RecA\(^-\) Z-mating occurred. One of the purified clones exhibited the markers CarB\(^2\), Leu\(^2\), Lac\(^+\), Gal\(^2\) and RecA\(^2\) of its F\(^-\) parent MG351. This was foreseeable. However, it yielded, at a very low frequency, Lac\(^-\) bacteria that did not share markers of its parents but those of an ‘ancestor’. Indeed, these Lac\(^-\) bacteria (1) required methionine instead of tryptophan, (2) were sensitive to FhuA-specific phages, unlike the FhuA\(^-\) H19-like strain, (3) were sensitive to both phages \(\lambda\) and Fig-specific \(\phi_m\), to which MG351 was respectively immune and resistant, and (4) were resistant to UV irradiation, to which both RecA\(^-\) H19-like and MG351-like strains were highly sensitive. In fact, these various features characterize strain MG401, which had been used two crosses upstream.

The occurrence of this apparently atavistic form was analysed in a random reisolate of the MG351-type. After single-cell isolation, a sample was first incubated in synthetic medium M9 with glucose, casein hydrolysate and uracil for balanced growth to occur. When the bacterial concentration reached \(10^5\) c.f.u. ml\(^{-1}\), the culture was tested and found to contain only one type of bacteria displaying the nutritional requirements, Lac\(^+\) character and UV sensitivity of MG351. A small aliquot of the culture (\(1\ ? 3 \times 10^5\) c.f.u.) was distributed in 12 tubes containing the same balanced synthetic medium. The 24 h subcultures were tested in two ways for the presence of MG401-like bacteria. Firstly,
 aliquots of each subculture were plated on MacConkey agar after UV treatment aimed at eliminating most of the RecA− cells. Secondly, untreated aliquots of each subculture were plated on MA+ methionine for the selection of MG401-like bacteria. Table 5 shows that both methods gave similar results: (i) among the red colonies formed on MacConkey agar by MG351-like bacteria surviving UV irradiation, pale colonies produced by Lac− MacConkey agar by MG351-like bacteria surviving UV irradiation, pale colonies produced by Lac− MacConkey agar by MG351-like bacteria surviving UV gave similar results: (i) among the red colonies formed on MG401-like bacteria. Table 5 shows that both methods apply to no prophage, is non-immune (Gratia, 1994). The same form was scant, almost null, because of their RecA− form, which was FhuA− LamB−. An asymmetrical behaviour is evident in Ncds formed using MG388, where only one chromosome (b) is carrying a prophage (λ); only the β form is immune to the phage, whereas the α form, which expresses chromosome a carrying no prophage, is non-immune (Gratia, 1994). The same applies to φ105 lysogens among B. subtilis Ncd exfusants (Guillén et al., 1983). Experiments were conducted to see how general the phenomenon was and whether it would shed light on the phenomenon of noncomplementing diploidy.

In Table 6, three derivatives of MG388a lysogenized by the hybrid phage φ80h80 are described. This strain was expected to carry a different prophage on each chromosome: φ80h80 inserted in att80 on chromosome a; λ in its site on chromosome b. Subclonal analysis shows how parental types and lysogeny for λ or φ80h80 segregated among first-order subclones of the purified clone [1], identified by chromosomal markers, or among higher-order subclones [2], [3]. It is noteworthy that whenever a lysogen converted from the initially selected parental form to the other, the prophage ceased to be apparent. This means that inactivation of the prophage-bearing chromosome leads to silencing of phage DNA functions, and notably to a return to the non-immune state. It also shows that lysogenization affects only the expressed chromosome. These observations were made possible because cross-infection was unlikely to occur: (i) λ was hardly produced at all by the RecA− cells of the β form, and (ii) the latter were Fig−, i.e. resistant to phage φ80h80 produced by the α form.

Subclone [3] is an example of what appeared to be a stabilized β form derived from a φ80h80 lysogen initially selected as an α-type colony (the same as in experiment [1]). On NA this subclone yielded only α-type colonies, but two late observations showed that chromosome a with its φ80h80

<table>
<thead>
<tr>
<th>Table 6. Subclonal analysis of lysogenic derivatives of strain MG388</th>
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<tbody>
<tr>
<td><strong>Analysed clone/subclone</strong></td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>1. MG388a [φ80h80]</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>2. Mixed subclone of clone 1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>3. β type subclone of clone 2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*Immunity was tested using various phages with a suitable host range: the β form, which was FhuA− Fig− LamB−, was tested against clear mutants of λ and hybrid derivative hλh80 (control of sensitivity to αvir); the α form, which was FhuA− Fig− LamB−, was tested against a clear mutant of φ80h80 (h8imm80c) (control of sensitivity to φ80h80vir).†Production of phages was tested after UV irradiation; the production of phages λ and φ80 by cells of the β form was scant, almost null, because of their RecA− phenotype. Infectious centres (IC) were observed on lawns of α-form bacteria selected on MA+ arginine + proline + 1% LB.
prophage were still present. Firstly, one sy colony of the β-form subclone plated on GDA displayed much delayed segregation of α-type bacteria (Table 6, analysed subclone 3, line 3); secondly, some colonies derived from subclone [3] failed to throw off any α-type bacteria but gave rise to φ80h+ infectious centres. The latter signalled the switch from expression of the β chromosome, which did not carry that prophage, to expression of chromosome α, which did, in a cytoplasm lacking the repressor of φ80-immunity, resulting in spontaneous development of φ80h+.

An interesting feature of lysogeny lies in the possibility to visualize a transient switch in phenotypically non-lysogenic isolates to the production of phage, detectable in the presence of non-immune surrounding cells, especially when the diploid character appears, by chance, on rare occasions. Tests described in Fig. 3 concerned strain MG393, derived from Z-mating between MG391 and MG392 (NaLA−). It behaved as an unstable Ncd x/β. After infection with φ80h+, only the MG391-like (x) form was lysogenized. The culture was streaked on NA containing nalidixic acid. Resistant bacteria formed colonies of the β form, which were apparently pure and, like MG392, were non-lysogenic (ly+), NaLA−, and required tryptophan. In spite of their lack of immunity to superinfection with φ80h+c, bacteria of the β form were believed to carry the silent genome of the α form with the φ80h+c (x+) prophage and also the wild-type nal+ allele. Nalidixic acid can induce prophage, including φ80h+c, an observation believed to be related to the ability to induce the SOS response (Drlica & Zhao, 1997). When drops of 50 µg nalidixic acid ml−1 were deposited on lawns of bacteria of the MG392-like β form growing on LB-agar, infectious centres appeared. This is interpreted as signalling induction of the φ80h+c prophage by nalidixic acid in cells resulting from a rare and/or transient switch to the x form (NaLA+ly+) and consecutive release of a few phage by the majority β non-immune bacteria. This happened in 12 of the 86 tested subclones and the frequency of infectious centres varied from 10−5 to 10−7 per 100 c.f.u. according to the isolates then proved to be Ncd [α ly+] /β ly−.

If there were cases of mere sticking of imperfectly isolated cells, production of phage by one member of a cluster would have persisted through reisolation cycles. This was not the case, because any sign of lysogeny (phage production or phage immunity) disappeared when isolates issued from crosses involving a strain carrying a prophage were subcultured. On the other hand, in various descendants of non-lysogenic clones, samples from lyophilized cultures continued to throw off lysogenic bacteria even after a considerable time had passed. The phage produced was of the same specificity as the parental prophage.

**DISCUSSION**

In agreement with the previously formulated conclusion (Gratia, 1994; Gratia & Thiry, 2003) that ‘spontaneous zygogenesis’ or ‘Z-mating’ fundamentally differs from

F-mediated conjugation, the present study shows that it generates diploidy, at first complementing and then more or less stably noncomplementing.

Persistent heterozygosis of markers widespread over the whole chromosome gives rise to phenotypic switching. Some subclones displaying a parental or a recombinant type turn out to be Ncds, like MG388, described previously (Gratia, 1994): one set of markers switches off, and a whole new set switches on. The switch included markers as varied as nutritional and fermentation characters, resistance or sensitivity to UV and to bacteriophages, as well as characters involved in lysogeny when derivatives were lysogenized.
Some biparental zygotes expressed a phenotype typical of one parent until they eventually switched to that of the other parent. Through experiments in which the phenotype of a given clone switched from one reisolation to the next, the tested markers of a parent appeared and disappeared simultaneously. In the case of MG388 or in products of mating between RecA strains, e.g. between H19-like and MG351 (see Results section 'Evidence for an ancestral silent genome in an Ncd'), switching was observed between all the parental markers spread along the entire chromosome. An attempt at representing what might have occurred in the descendants of cells resulting from a Z-mating is shown in Fig. 4.

Such behaviour resembles that of some exfusants of several species of Bacillus, which were interpreted as Ncds by Hotchkiss & Gabor (1980) and extensively analysed by the Orsay group, which confirmed this assumption (Guillén et al., 1983; Lévi-Meyrueis & Sanchez-Rivas, 1984; Fleisher & Vary, 1985; Guillén et al., 1985; Le Dérouet et al., 1992; Grandjean et al., 1996a, b, 1998). The evidence for noncomplementing diploidy among B. subtilis exfusants is strong despite controversy (Sanchez-Rivas & Lévi-Meyrueis, 1994 vs Hauser & Karamata, 1992). The problem is that exfusants may evolve into true haploid recombinants, phenotypically indistinguishable from Ncds, which often express the same chromosome for many generations.

The work on B. subtilis exfusants (Lévi-Meyrueis & Sanchez-Rivas, 1984) suggests that inactivation of one parental genome might occur very early in the evolution of the initial mating product. The data obtained here in the fluctuation test (Table 2) and respreading experiments (Table 3, Fig. 2) suggest that biparental zygotes exceed by far the frequency of colonies of more or less stable complementing diploids expressing prototrophy (approx. $10^{-4}$ per minority parent cell). The same conclusion can be drawn from the increased frequencies of non-viable cells detected by the epifluorescence staining method. The reproducible differences in such frequencies between mating mixtures or, better, the selected Z-mating products, and the parents indicate the relatively high amount of cells having lost an active genome. Accordingly, the frequency of putative fusion products detectable by immunofluorescence microscopy with BrdU labelling of the F- partner (Gratia & Thiry, 2003) also exceeds by far the frequency of complementing clones selected as prototrophic colonies.

In the present case, as in the case of B. subtilis exfusants, the switch from one mode of expression to the other for all the genes marked in parents indicates that the other chromosome was present all along, albeit silent. There may have been recombination between parental markers, but the data cannot be explained without accepting that at some point in time both parental genomes were present, whole,

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**Fig. 4.** Schematic representation of possible events following Z-mating. Segregation of colony-forming cells carrying parental and/or recombinant markers and non-viable haploid cells carrying an inactive chromosome. *Reactivation of p2 chromosome in noncomplementing diploid of P1 phenotype.*
inside the same cell. Noncomplementing diploidy, responsible for changes from one form to the other, at least in some subclones, demonstrated the occurrence of genotypic mixing in the cases where recombination was hindered or prevented, i.e. between RecA⁻ Szp⁺ H19-like and F⁻ MG351 strains.

To estimate the rate of Ncd formation would require a means for unmasking bacteria which stably masquerade as haploids. One approach might be to detect events signaling that the silent genome has been reactivated, even transiently. Such an event occurs when the silent chromosome carries a prophage, λ or φ80 (Gratia, 1994; this work): lysogeny is switched off until the chromosome is reactivated, with resultant phage development at the expense of sensitive bacteria present in the culture. The same was observed previously in the case of φ105 in B. subtilis (Guillén et al., 1983).

When a given form of an unstable isolate was mated with an F⁻ (non-Szp⁺) strain, the possibility arises that some phenotypically diploid or haploid Z-mating products might in fact have been triploids expressing only one or two genomes. That triploidy can occur was revealed in some cases (Table 5), but its occurrence in all matings remains to be determined. Crosses between three parents might be undertaken in further experiments designed to examine this issue. In this paper, the observation is proposed to mean that a stably silent genome may persist throughout the life of a strain subjected to successive crosses. The presence of an ancestral silent chromosome is revealed through recombination with an active genome or reactivation by an undetermined mechanism.

If no switch is detectable, even over a long period, one could imagine that the Z-mating-derived strain has undergone true genotypic haploidization or permanent inactivation of one chromosome resulting in undetectable non-viable segregants. Perhaps one chromosome has become severely defective because of extensive deleterious deletions.

The occurrence of genotypic mixing of two strains resulting from induced fusion of protoplasts or spontaneous zygogenesis leads to other questions. In diploids, are the two parental replicons able to replicate more or less synchronously? Would the situation be similar to that of a cell with two copies of its chromosome, in which one copy has undergone a mutation? If the mutation is recessive, it is usually considered that segregation is required for its expression. One might then wonder whether this really happened or whether inactivation of the wild-type chromosome occurred. In other words, might diploidy, or rather noncomplementing diploidy, be more frequent than presently believed?

In the descendants of Z-mating products recombination events appeared to be infrequent, limiting the accumulation of different recombinants in mixed subclones. In the case of MG388, recombination between chromosomal wild-type alleles in trans was seldom observed (J. P. Gratia, unpublished). In noncomplementing exfusants (Ncds) of B. subtilis, silent DNA appears to display abnormally low transforming activity, which means that recombination between the active and the inactive chromosome is infrequent (Guillén et al., 1985). The present experiments of infection with phages, to which the two forms of MG388 (Table 6) had distinct receptors, indicate that site-specific recombination, such as insertion of a phage genome, also appeared not to take place in the silenced chromosome, at least at a frequency that would make such recombination easily detectable.

The inactivation of entire replicons, such as plasmids or chromosomes, is a very important problem reminiscent of the phenomenon described in mice 40 years ago (Lyon, 1993). Reversible silencing of all or part of a chromosome has also been observed in other eukaryotes, such as insects (Crousi et al., 1971) and plants (Assaad et al., 1993). The term ‘epigenetic’ is now often used to describe a reversible modification of genetic expression that can be transmitted to the progeny without any alteration in the DNA sequence (Holliday, 1994; Lee & Jaenisch, 1997). Thaler et al. (1990) and Grandjean et al. (1996b) drew a parallel between noncomplementing diploidy in B. subtilis and X chromosome inactivation. As regards Z-mating products, a better understanding of the mechanism involved is required before extending any comparison between noncomplementing diploidy in E. coli and eukaryotic chromosome silencing. In spite of similarities between the observations described here and the reported studies on B. subtilis, the situation might be different, as E. coli and B. subtilis are highly divergent bacterial species. Therefore Z-mating is planned to be investigated in terms of molecular biology in the same way as B. subtilis exfusants were analysed.

In conclusion, noncomplementing diploidy in bacteria is not limited to diploids formed by fusion of protoplasts of, for example, B. subtilis. Further investigation of this phenomenon in bacteria is likely to contribute to the elucidation of the mechanisms of chromosome silencing in general.

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